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Partitioning Invertase Between a Dilute Water Solution and Generated Droplets

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Abstract

Water droplets or mist occur naturally in the air at seashores. These water droplets carry inorganic and organic substances from the sea to the land via the air, creating fertile land in sandy coastal areas *(1)*. The same phenomenon occurs in an air-fluidized bed bioreactor *(2)*. In an air-fluidized bed reactor, proteins can be transferred from the bioreactor semisolid bulk phase to an enriched droplet phase. This protein transfer process (droplet fractionation) can be experimentally simulated by shaking a separatory funnel containing a dilute solution of a given protein, which can be an enzyme like invertase. The created droplets become richer in invertase (protein) than that of the original dilute solution. The droplets can then be coalesced by trapping them and recovering the concentrated protein in the new liquid phase. Typically, in such a droplet fractionation process a collected enzyme can be degraded in its ability to catalyze a chemical reaction. In this article, we explore whether the initial solution pH control variable can be adjusted to minimize the decrease of enzyme activity in this process. The protein droplet recovery problem is one in which the recovered amount of desired protein (enzyme) in the droplet is maximized, subject to the minimization of the enzyme activity loss. The partition coefficient, which is the ratio between the protein concentration in the droplets and the residual solution, is maximized at approx 4.8 and occurs at pH 3.0. Here, the partition coefficient for invertase decreases as the initial solution pH increases, between pH 3.0 and 8.0. Interestingly, the initial solution surface tension seems to be inversely proportional to the partition coefficient. The partition coefficient reaches a maximum value at a surface tension value of approx 63 mN/m at pH 3.0. The enzymatic activity of the initial, the residual, and the droplet solutions all decrease as the bulk solution pH increases. A decrease of enzymatic activity was observed in the residual bulk solution when compared with that in the initial bulk solution at all pH levels. Also, up to 90% of the invertase activity was lost in the droplets when compared to the initial bulk solution.

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Index Entries: Droplet fractionation; droplets; invertase; protein separation; decrease in enzyme activity.

Introduction

Invertase has many applications and uses in the food industry, such the ability to produce fructose syrup from a sucrose solution, softening chocolates, and keeping food products moist *(3)*. A common source of invertase is from the outer cell wall of *Saccharomyces* yeast *(4)*. Typically, the purification procedure for recovering invertase is a prolonged one of autolysis, heat treatment, salting out, precipitation, and adsorption. The prolonged autolysis technique collects invertase by precipitation (a salting out technique) in a 70% saturated ammonium sulfate solution *(4)*. The salting out method works well in the laboratory for precipitating invertase; however, this technique can be costly and difficult to apply at the industrial level since it requires the subsequent removal of the contaminating salt and its disposal as waste product. New separation techniques, such as droplet fractionation and foam fractionation, may provide an alternative initial method for concentrating and separating desired proteins, such as invertase, cellulase, and amylase, from dilute enzyme solutions. Such methods appear to be promising early steps for enzyme purification.

A previous study has shown that a specific protein (within a mixture of proteins) is carried over in an effluent air stream from an air-fluidized bed *(5)*. That study led to the idea of separating certain proteins using formed droplets from a dilute protein solution by trapping those proteins in the water droplets, which are carried in the air phase. The carried protein in the droplets can then be recovered by coalescing the droplets at a temperature below 0°C *(6)*. This proposed droplet separation technique for enriching proteins was implemented in a 600-mL separatory funnel by gentle inversion of that funnel *(6,7)*. The partition coefficient, K_p, the ratio of the protein concentration in the droplet phase to that in the residual bulk phase, was determined to quantify those studies. In that initial study using a crude invertase solution, it was found that the partition coefficient corresponding to the noncentrifuged bulk dilute solution was about 47.5 (the protein concentration in the droplets was 47.5 times higher than that in the residual solution). Since the partition coefficient obtained in this study is closer to 3, it may be that the droplet technique is even more suitable for recovering the accompanying cell debris (the protein impurities from the crude enzyme mixture, which are likely to make up the remaining protein in the droplets).

Droplet fractionation may be a promising technique for separating invertase (or contaminants in a crude invertase mixture) from a dilute invertase solution. Like in foam fractionation, the bulk solution pH may play a key role in partitioning invertase from the dilute solution. Since the bulk solution pH seems to be strongly correlated with surface tension and is often easier to measure, the bulk solution pH could be used as an indirect

measurement for controlling the surface tension. In this droplet fractionation study, the bulk solution pH is both easy to measure and is an important regulatory variable. Therefore, it is selected as the main parameter here to investigate the effect of the surface tension, the partition coefficient, and the enzymatic activity of invertase in a droplet fractionation process.

Materials and Methods

Yeast invertase (lot no. I-9253), sodium hydroxide (lot no. 873487), and sodium citrate (lot no. 66F-0345) were purchased from Sigma (St. Louis, MO). Coomassie brilliant blue G-250 (lot no. 23242) was purchased from Bio-Rad (Richmond, CA). Sodium sulfite (lot no. 785778), citric acid (lot no. 795790), glucose (lot no. 793019), 85% *o*-phosphoric acid (lot no. 952498), 95% ethyl alcohol (lot no. 952264), and sucrose (lot no. 771722) were purchased from Fisher Scientific (Fair Lawn, NJ). Dinitrosalicylic acid (lot no. 09026LW) was purchased from Aldrich Chemical Company (Milwaukee, WI). Phenol (lot no. 250G) was purchased from Fluka (Milwaukee, WI).

Experimental Procedure

Invertase solution (approx 100 mg/L) was prepared by dissolving invertase granules into deionized water followed by centrifugation (Marathon 21K, Fisher Scientific) at 1073 g for 10 min. The supernatant was collected, kept in a refrigerator at 8°C, and used within 1 wk. The schematic of the droplet fractionation procedure is shown in ref. *2*. The experiments followed a previously described procedure *(6)* using a separatory funnel. The invertase solution initially filled up the 600-mL separatory funnel to the top of its neck in order to expel air containing dust and other contaminating proteins. After that a cotton plug was placed at the top of the funnel to prevent outside air dust from reentering the funnel. The invertase solution was then drained from the funnel to the desired experimental volume of 100 mL. The air above the liquid space was presumed to be clean enough for these experiments. It is noted that previous checks with distilled water in the funnel confirmed that the contaminating protein was negligible *(6)*. The cotton plug was then immediately replaced by a glass stopper in order to confine the liquid and droplets when the funnel was shaken. Droplets were generated in the glass funnel by manually repeating the inversion of the funnel for 10 min (each inversion takes approx 3 s). After the inversions, a cotton plug was placed back at the top of the funnel and the residual solution was drained. The stopcock valve was shut immediately after the solution was drained. The funnel was then cooled for 30 min in a freezer at –18°C to collect the droplets. The volume of collected droplets was 1 ± 0.15 mL. It was assumed to be 1 mL for all experiments to average out the effect of liquid sticking to the glass funnel and to the glass graduated measuring cylinder. This 15% random volumetric variation, thus, could contribute to the variation in partition coefficient and activity measurements. The collected volume (approx 1 mL) of droplets (containing highly concen-

trated protein) was diluted to the desired volume of 5 mL for the total protein and the invertase activity assays (3 mL for the total protein and 1.5 mL for the invertase activity). The initial, residual, and collected droplet solutions were all assayed for the total protein content and invertase activity.

The effect of pH on the droplet fractionation of invertase was studied for an initial protein concentration fixed at approx 100 mg/L. 1 *N* sodium hydroxide and 1 *N* hydrochloric acid were used for adjusting the pH of the initial invertase solution in the range between 2.0 and 12.0. This addition of 5–10 drops (maximum) is less than a 0.001-mol addition of acid or base to 600 mL solution. Additions of NaCl <0.1 mol raise the surface tension of water <1% *(8)*. Hence, the additions of acid or base are likely to have a negligible effect on the measured surface tension. In addition, the surface tension of invertase solution was determined as a function of the bulk solution pH using the Wilhelmy Plate method (SKV Instruments Ltd., Helsinki, Finland).

Total Protein Assay

The Coomassie blue (Bradford) method was used to determine the total protein content in the invertase solution *(9)*. In all of these assays, 3 mL of sample and 2 mL of Coomassie blue reagent were used in conjunction with a Bausch and Lomb Spectronic 20 spectrophotometer set at 595 nm. The optical absorbance of each sample was read after the Coomassie blue reagent was added to the invertase solution for 5 min. The following linear equation correlating data for this invertase assay was used as the calibration curve for the invertase solution:

$$\text{Invertase (protein) concentration (mg/L)} = 1429 \cdot (\text{absorbance @ 595 nm}) \quad (1)$$

Invertase Activity Assay

Sucrose solution (100 mg/L) was used as the invertase enzyme substrate and 0.05 *M* sodium citrate solution was used as the buffer solution (pH 4.7). Invertase sample solution (1.5 mL), sucrose solution (0.5 mL), and buffer solution (1 mL) were added to a 10-mL test tube. This solution was then incubated in a dry-bath incubator (Fisher Scientific) at 37°C for 15 min. After incubation, the enzymatic reaction of invertase was stopped by cooling the test tubes in an ice bath. Three milliliters of dinitrosalicylic acid (DNS) reagent *(10)* was added to the test tube containing 3 mL of the digested sucrose solution. The test tube was then placed in a boiling water bath for 5 min. After that, the test tube was removed and cooled in running water to adjust the solution to room temperature. The optical absorbance of the solution was determined at 575 nm wavelength using a Bausch and Lomb Spectronic 20 spectrometer. The invertase activity was determined in terms of the rate of reducing sugar (glucose) released per mass of invertase

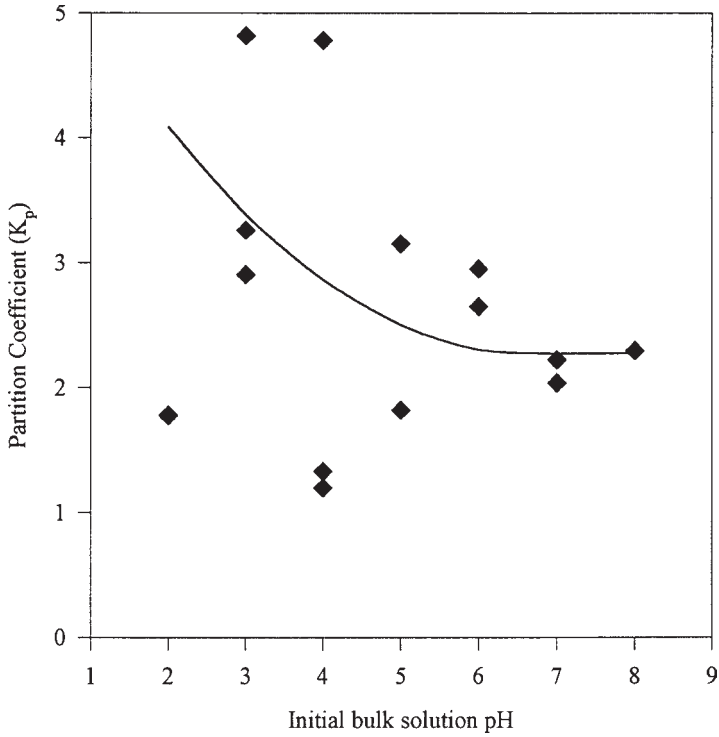

Fig. 1. Effect of initial bulk solution pH on the partition coefficient of invertase solution at the initial protein concentration of 100 mg/L.

per minute. The released glucose was determined from the linear calibration curve in terms of the reducing sugar concentration *(11)*:

$$\text{Reducing sugar concentration (mg/L)} = 37.6 + 395 \cdot (\text{absorbance @ 575 nm}) \quad (2)$$

Results and Discussion

The K_p is defined as the ratio of the protein concentration in the droplets ($C_{Droplet}$) to the remaining protein concentration in the residual solution ($C_{Residue}$):

$$K_p = (C_{Droplet})/(C_{Residue}) \quad (3)$$

Implicit in the interpretation of K_p is the assumption that no protein remains on the glass separatory funnel wall, so all of the protein leaving the bulk is presumed to transfer to the droplets.

Figure 1 illustrates the effect of the initial bulk pH on the K_p. The K_p dropped about in half when the initial bulk solution pH increased from 2.0 to 7.0. In this study, the K_p values ranged from 1.3 to 5, the latter being about 1/10 that of the previous study of $K_p = 47.5$ *(6)*. Considering the fact that the

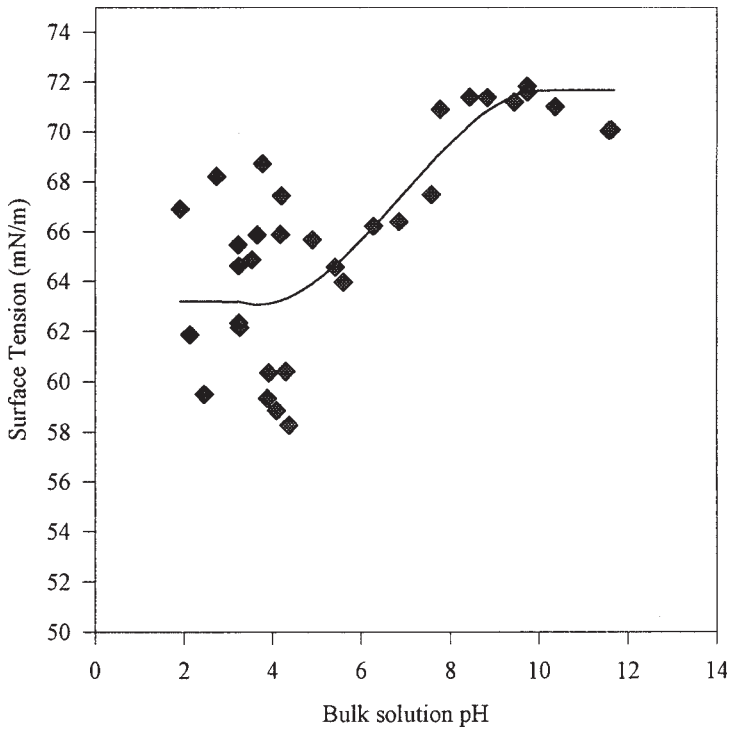

Fig. 2. Effect of initial bulk solution pH on the surface tension of invertase solution at the initial protein concentration of 100 mg/L.

previous work used impure and noncentrifuged invertase for the experiments, it is suspected that the cell debris impurities, with attached proteins, also carried over to the droplets, resulting in the high droplet protein concentration. Typically, here, more than 90% of the noncentrifuged crude invertase that was used was observed to be in the invertase solution as suspended solid particles before centrifuging. If confirmed by a subsequent study, these two different experiments can be the basis for suggesting that droplet fractionation may be a useful method for initially removing cell fragments from invertase.

Surface tension generally plays an important role in a foam fractionation process *(12)*. Similarly, the surface tension may also be an important parameter in a droplet fractionation process. The surface tension of a protein solution generally is a function of the bulk solution pH, as shown in Fig. 2 for a 100 mg/L invertase solution. The minimum surface tension of this invertase solution is approx 62 m*N*/m, at pH 4.0, which is close to the invertase isoelectric point (pI = 4) *(3)*. At the bulk solution pH between 8.0 and 10.0, the surface tension is approx 70 m*N*/m, which is close to the surface tension of water (72 m*N*/m). Thus, the invertase solution at high pH is hydrophilic. In contrast, invertase at low pH behaves much more like a hydrophobic solution because it has a lower surface tension.

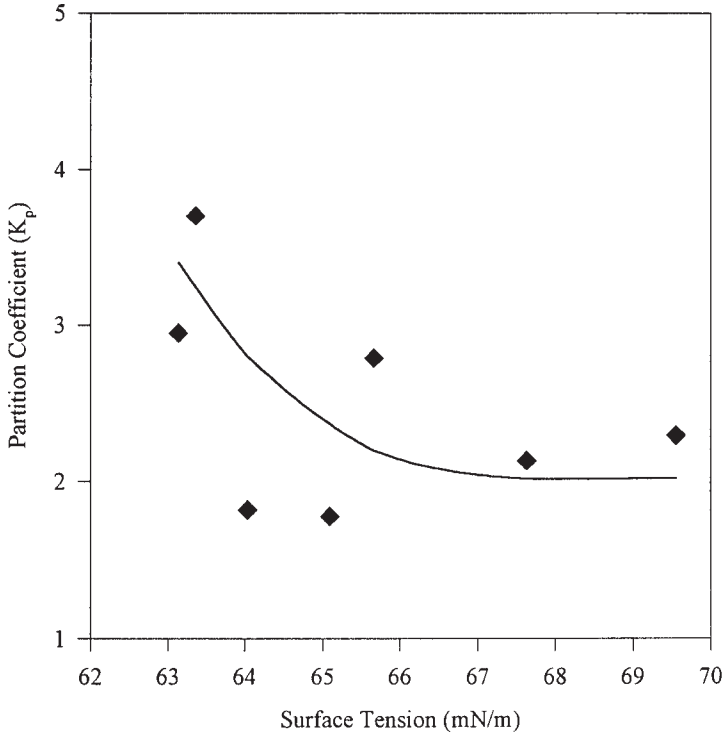

Fig. 3. Effect of surface tension on the partition coefficient of invertase solution at the initial protein concentration of 100 mg/L.

The surface tension is correlated approximately linearly (with a large standard deviation around pH 2.0 to 7.0) with the bulk solution pH for the invertase solution. The K_p vs the surface tension trajectory shown in Fig. 2 may provide a way of predicting the degree of invertase partitioning from the hydrophobicity of invertase (the surface tension is inversely related to the hydrophobicity). As shown in Fig. 3, for the data in Fig. 2, the K_p of the invertase appears to decrease (within experimental error) as the surface tension increases, reaching a minimum value for K_p of around 2 at a surface tension value above 67 m*N*/m at bulk solution pH >8.0. This is another way of saying that the partition coefficient decreases as the hydrophobicity decreases.

Figures 4 and 5 show the effect of the initial bulk solution pH on the invertase specific activity of the initial solution, and the residual and droplet solutions, respectively. The invertase specific activity of the initial solution ranged between 0.05 and 0.11 (mg sugar/min)/mg protein and the invertase specific activity for at least some of the points is minimized at approx pH 5.0 (close to the pI of 4). The invertase specific activity of the droplets ranged between 0.009 and 0.05 (mg sugar/min)/mg protein and also had a minimum of approx pH 5.0. The error about the mean values in the calibration curve is about ±10%. The errors observed in Figs. 4 and 5 are

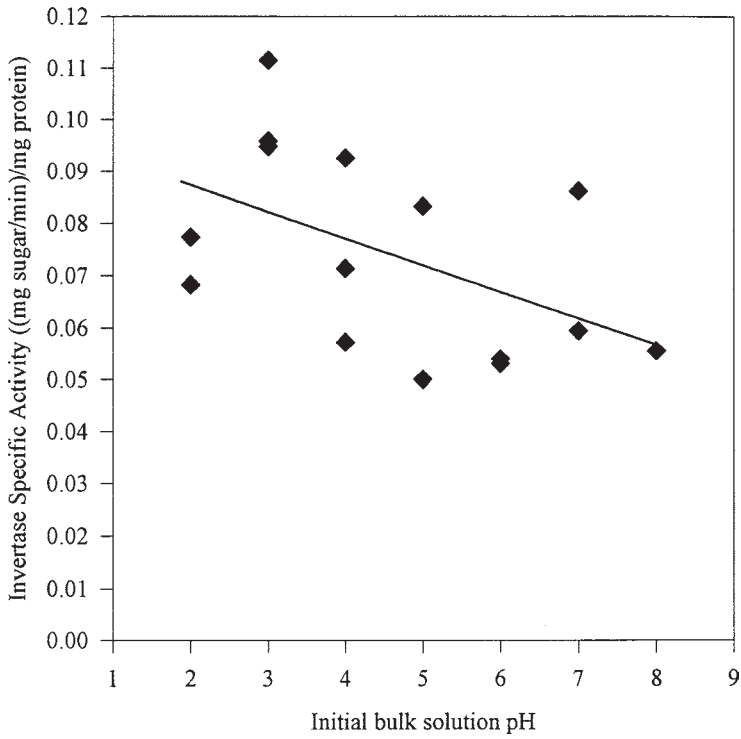

Fig. 4. Effect of initial bulk solution pH on the invertase specific activity of initial solution at the initial protein concentration of 100 mg/L.

about ±20%, meaning that half of that error can be attributed to the droplet experiment itself. The invertase specific activity of the droplets dropped to about half of the initial solution at pH 2.0 and close to a negligible value for pH above 5.0. The approx 50% decrease in activity may be caused by the processing conditions, e.g., the invertase in the droplets may be damaged during droplet fractionation because of the shear forces occurring during the protein transfer process. The invertase specific activity of the residue ranged between 0.08 and 0.16 (mg sugar/min)/mg protein (neglecting the unusually high values at pH 3.0) and decreased significantly as the initial bulk solution pH increased. At an initial bulk solution pH between 7.0 and 8.0, the invertase specific activity in the residue equalled that of the initial solution. Below pH 7.0 the residual enzyme activity may even be slightly greater that the initial bulk solution value. This latter observation needs to be confirmed by additional experiments to determine whether this increase is real and not the result of experimental error.

Conclusions

Droplet fractionation of the invertase dilute solution can concentrate proteins relative to the residual solution into droplets from 1.3 to 3.7 times

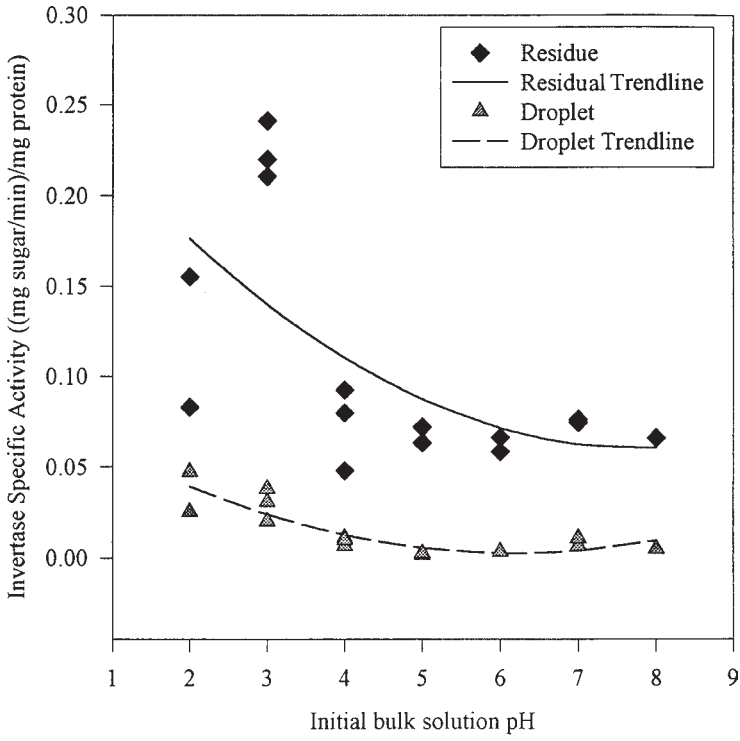

Fig. 5. Effect of initial bulk solution pH on the invertase specific activity of residual and droplet solutions at the initial protein concentration of 100 mg/L.

the K_p values within the range of initial bulk solution pH of 2.0–12.0 (at a total initial protein concentration of 100 mg/L). Increasing the initial bulk solution pH reduces the partition coefficient. The maximum partition coefficient of 4.8 occurred at initial bulk solution pH of 3.0. Since the surface tension of the invertase solution increases as the initial bulk solution pH increases, the partition coefficient also decreases when the surface tension of the invertase solution increases. Within experimental error, below pH 2.0 (and at surface tensions above 67 mN/m), the partition coefficient reaches minimum values. The specific activities of invertase for the initial, residual, and droplet solutions all decrease when the initial bulk solution pH increases. The invertase specific activity in the droplets is reduced to about half the initial solution activity at pH 2.0 and even more for higher pH. It appears that the invertase specific activity of the residual solution may even be higher than that of initial solution, but this observation must be confirmed with further experiments. The droplet fractionation of invertase solution may provide a method to remove cell debris from crude invertase rather than concentrating the invertase itself, as was surmised. The inferred observation between this and a previous study must be checked with direct droplet fractionation experiments using only cell wall debris.

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